FRACTIONATION OF RAT HIGH DENSITY LIPOPROTEIN BY AFFINITY CHROMATOGRAPHY

Effect of prolonged ultracentrifugation on high density lipoprotein subspecies

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1. Introduction

Serum lipoproteins are usually prepared by sequential flotation at increasing solvent density in the ultracentrifuge [1]. It is known, however, that lipoproteins so prepared are modified during ultracentrifugation [2]. Rat HDL, isolated between densities 1.065 kg/l and 1.21 kg/l, contain several apolipoproteins [3,4] of which apo A-I (mol. wt 28 000), apo ARP (mol. wt 35 000) and apo A-IV (mol. wt 46 000) have been detected in the lipoprotein free d = 1.21 kg/l infranatant [5,8]. Of these, apo ARP appears in the greatest quantity and its appearance is dependant upon the force and duration of centrifugation [5,8]. This apo ARP may not be homogeneously distributed in rat HDL fractionated by gel filtration [19].

In this study, affinity chromatography of rat HDL using heparin-Sepharose has been employed to isolate an apo ARP-enriched HDL and to examine the effect of ultracentrifugation on this HDL.

Abbreviations: HDL, high density lipoproteins; HDL_S, HDL prepared by a short ultracentrifugation; HDL_L, HDL prepared by a long ultracentrifugation; apo A-I, apolipoprotein A-I; apo ARP, arginine-rich apolipoprotein; apo A-IV, apolipoprotein A-IV; apo C, C apolipoproteins; SDS, sodium dodecyl sulphate

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2. Materials and methods

2.1. *Animals*

Male Lister rats (250–350 g) allowed free access to a standard laboratory diet were exsanguinated under ether anaesthesia. Pooled serum from 10–15 rats, to which was added sodium azide (0.1 g/l) and thiomersal (0.05 g/l) was used.

2.2. *Ultracentrifugation*

Ultracentrifugation was performed at 105 000 × g in a type 40 rotor at 16°C using a Beckman Spinco L2-65B ultracentrifuge. Lipoproteins were isolated by tube slicing. Pooled serum was adjusted to d = 1.065 kg/l and the $d < 1.065$ kg/l lipoproteins removed by centrifugation for 18 h. The HDL were then isolated after 18 h centrifugation at d = 1.21 kg/l. Half of these HDL (HDL_S) were then centrifuged for a further 72 h at d = 1.21 kg/l and te HDL (HDL_L) and infranatants recovered. Density adjustments were made by adding either solid NaBr (exp. 1) or 400 g/l NaBr (exp. 2).

2.3. *Affinity chromatography*

Heparin–Sepharose was prepared by covalent coupling of heparin to Sepharose 4B, activated with cyanogen bromide [10,11]. Samples were applied to a heparin–Sepharose column (1 × 12.5 cm) equilibrated with 2 mM sodium phosphate, pH 7.4, containing 0.05 M NaCl [12] and all non-binding material eluted at 10 ml/h with the same buffer. Heparin-bound material was then eluted with 2 mM

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sodium phosphate, pH 7.4, containing 1 M NaCl and dialysed against an appropriate volume of 2 mM sodium phosphate, pH 7.4, to reduce NaCl to 0.05 M.

2.4. *Delipidization*

Lipoproteins were delipidized with ethanol/diethyl ether, 3:2 (v/v) at –20°C [13].

2.5. *Polyacrylamide gel electrophoresis*

Polyacrylamide gels containing a 4–30% linear gradient of acrylamide were obtained from Pharmacia Ltd, London. Electrophoresis was performed at 125 V in Tris/glycine buffer, pH 8.2 [14]. SDS–polyacrylamide gel electrophoresis was carried out on 10% acrylamide gels [15].

2.6. *Immunological methods*

Antisera to whole rat serum, apo A-I and apo ARP were raised in rabbits. Double diffusion methods were used to examine lipoprotein and apolipoprotein-containing fractions [16].

2.7. *Protein*

Elution of protein from columns was monitored at $A_{280\,nm}$. A modification of the methods in [17,18] was used to quantitate protein, using bovine serum albumin as standard.

3. Results

Affinity chromatography of rat HDL on heparin–Sepharose produced a non-bound and a heparin-bound fraction for all preparations of HDL investigated. Immunodiffusion showed that both fractions contained apo A-I and apo ARP and indicated that there was an enrichment of apo ARP relative to apo A-I in the heparin-bound fraction. SDS–polyacrylamide gel electrophoresis of the delipidized fractions obtained from HDL_S (fig.1) produced bands that were identified as apo A-I and apo ARP (as well

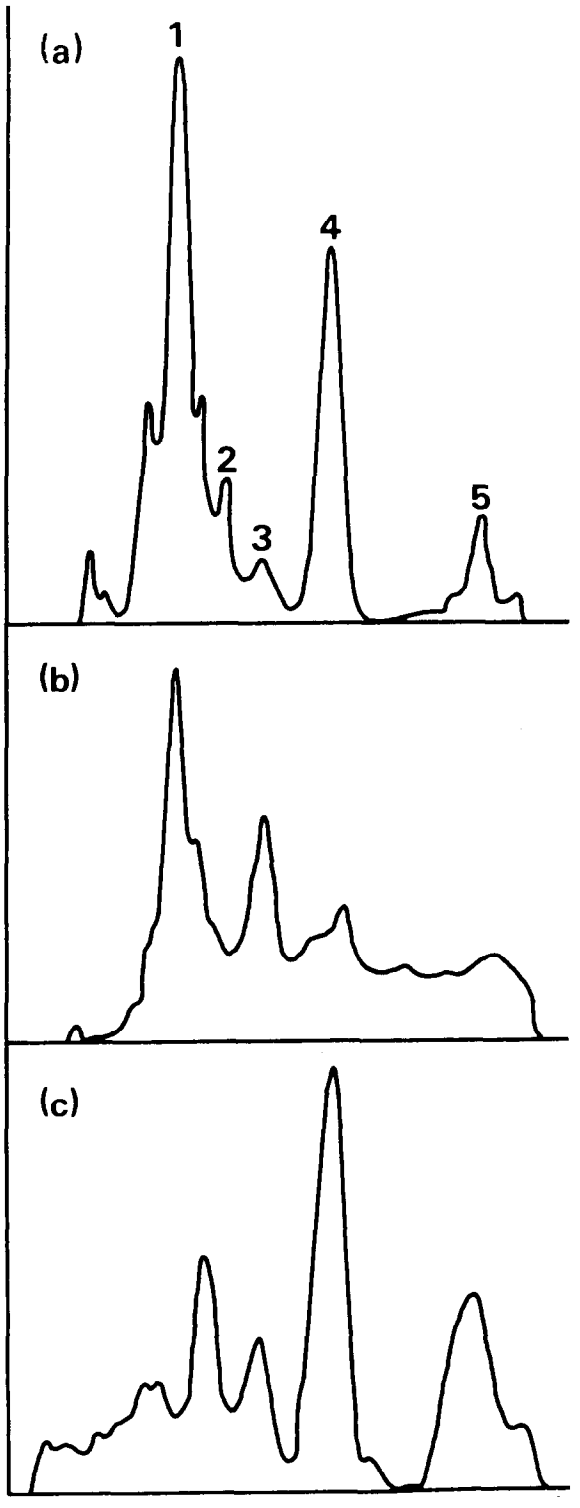

Fig.1. Densitometric scans of delipidized HDL_S fractions separated on 10% SDS–polyacrylamide gels: (a) non-bound HDL_S; (b) heparin-bound HDL_S; (c) reference sample of rat HDL. Numbers 1–5 indicate the positions of albumin, apo A-IV, apo ARP, apo A-I and apo C, respectively.

Table 1
Distribution of apo ARP and apo A-I in rat HDL fractionated on heparin–Sepharose

	apo ARP/apo A-I in HDL species	
	Non-bound	Heparin-bound
HDL_S	0.11 (3)	1.94 (3)
HDL_L	0.10 (2)	1.08 (2)

The values given represent the means of the apo ARP : apo A-I ratios for the number of experiments in brackets. These were calculated from measurements of the area under each peak, duplicate gels being analysed in each experiment

as apo C) on the basis of their molecular weight and immunological reactivity against specific antisera. Table 1 shows that the ratio of apo ARP to apo A-I was 18-times greater in the heparin-bound HDL_S than in the non-bound HDL_S. Analysis by gradient polyacrylamide gel electrophoresis (fig.2) revealed that the heparin-bound HDL_S was of larger particle size than the non-bound material.

The gradient gel analysis also revealed that the serum proteins were fractionated by affinity chromatography. The fractionation of serum proteins was due to specific protein–heparin and not protein–lipoprotein interactions as lipoprotein-free rat serum gave a similar distribution (not shown).

The changes in protein content between HDL_L and HDL_S produced by prolonged ultracentrifugation are shown in table 2. There was a large decrease in the amount of protein recovered in HDL_L, the heparin-bound HDL being more affected by the ultracentrifugation than the non-bound HDL. This decrease is attributable both to the removal of serum proteins from the HDL (see fig.2) and to the selective removal of apo ARP from the HDL particle as evidenced by the greater apo ARP : apo A-I ratio in the heparin-bound HDL_S compared with the heparin-bound HDL_L (table 1). The difference in the two experiments is due in large part to the different methods used in adjusting the serum density which affects the serum protein concentration in the ultracentrifuge tube. Although much reduced in quantity, the heparin-bound HDL_L was still of larger particle size than the non-bound HDL_L.

The infranatant derived from prolonged ultracentrifugation of HDL_S could also be fractionated

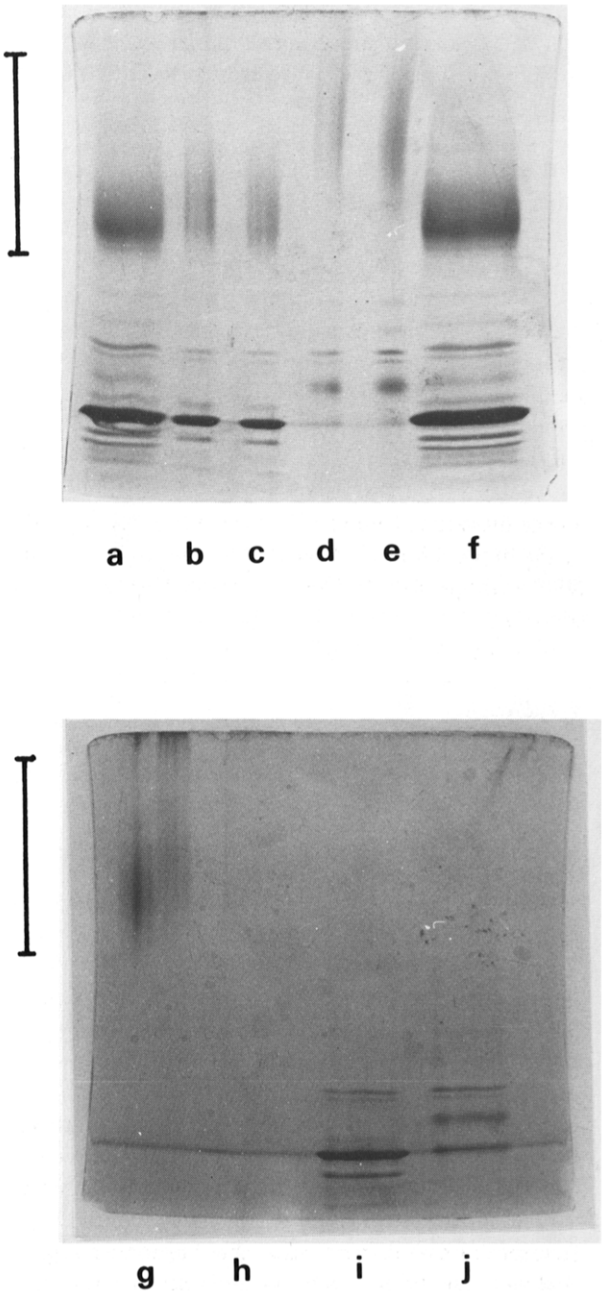

Fig.2. Gradient polyacrylamide (4–30%) electrophoretograms of subfractions of rat HDL from heparin–Sepharose chromatography: (a,f) HDL_S; (b,c) non-bound HDL_S; (d,e) heparin-bound HDL_S; (g) non-bound HDL_L; (h) heparin-bound HDL_L; (i) non-bound d = 1.21 kg/l infranatant; (j) heparin-bound d = 1.21 kg/l infranatant. Bar indicates position of HDL.

Table 2
Distribution of protein after fractionation of rat HDL on heparin–Sepharose

	Total protein			Heparin-bound protein		
	HDL_S	HDL_L	HDL_L / HDL_S	HDL_S	HDL_L	HDL_L / HDL_S
	(mg/dl)			(mg/dl)		
Exp. 1	166	42.3	0.25	14.9	2.3	0.15
Exp. 2	59	40.5	0.69	8.4	3.1	0.37

into non-bound and heparin-bound fractions. Immunodiffusion studies revealed that apo A-I was present only in the non-bound fractions and apo ARP only in the heparin-bound fraction. No band of molecular weight less than that of albumin was visible on gradient polyacrylamide gel electrophoresis of the heparin-bound infranatant suggesting that apo ARP is not present in a momomeric form in free solution.

4. Discussion

This work has shown that rat HDL can be separated into two species by affinity chromatography on heparin–Sepharose. It has been reported that human apo ARP is responsible for the binding of lipoproteins to heparin agarose [12]. The observation that apo ARP is enriched in heparin-bound rat HDL and is the only apolipoprotein of delipidized rat HDL that binds to heparin–Sepharose would suggest that the same mechanism is responsible for the binding of rat HDL to heparin–Sepharose. Several other protein species in rat serum are also bound to heparin–Sepharose and this could prove useful in purification studies.

The effect of prolonged ultracentrifugation on rat HDL illustrates the difficulties involved in preparing lipoproteins by this method. A short ultracentrifugation produces a more native product but one contaminated by other proteins. However, removal of the contamination by prolonged ultracentrifugation, dissociates apolipoproteins from HDL [8], especially from apo ARP-enriched HDL.

The heparin-bound, apo ARP-enriched HDL described here which has a larger molecular size than the non-bound HDL is in agreement with other data from gel filtration studies [9]. However, the apo ARP-enriched HDL still contains apo A-I and apo C which suggests it differs from the HDL in [19,20]. The latter studies however would have underestimated the amount of the heparin-bound HDL because of the ultracentrifugation involved.

The results of these investigations therefore further suggest that isolation procedures which avoid the use of the ultracentrifuge are necessary for detailed studies on the synthesis and metabolism of lipoproteins.

Acknowledgements

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